Meeting report

Cancer and kinases: reports from the front line

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A report on the Keystone Symposium 'Cancer and Kinases: Lessons from the Clinic', Santa Fe, USA, 14-19 February 2006.

The success of the Bcr-Abl kinase inhibitor imatinib (Gleevec) in treating chronic myelogenous leukemia (CML) has provided considerable impetus to the development of kinase-targeted therapies for solid tumors and hematological malignancies. A Keystone Symposium in February on the challenges of kinase-targeted drug discovery in oncology brought together an eclectic group of innovators and encompassed a broad array of topics in this field.

Identification of potential therapeutic targets

Cancer cells exhibit numerous mutations, many of which are in protein kinase genes, and one challenge is to distinguish which of these mutations are actually responsible for malignant transformation and thus might represent therapeutic targets. Michael Stratton (Wellcome Trust Sanger Institute, Hinxton, UK) described how high-throughput sequencing of cancer-cell genomes is being used to construct mutational profiles of human cancers. He described how somatic mutations acquired during gestation and throughout life culminate in tumors that bear mutations in oncoproteins and in DNA repair proteins. Sequencing of around 200 distinct types of cancer revealed that approximately 50% bear mutations in protein kinase genes, only a small number of which are thought to be responsible for malignancy. For colorectal and gastric cancers, the vast majority exhibit somatic mutations in kinase genes. Stratton's conclusion was that sequencing many examples of each rigorously characterized tumor type will be needed to permit the statistically significant identification of the 'driver' mutations that contribute to cancer.

Another approach to identifying protein kinases that might be therapeutic targets is to determine the effects of suppressing their expression using RNA interference (RNAi). William Hahn (Dana Farber Cancer Institute, Boston, USA) showed the power of this technique to suppress specific genes in mammals such as mice and humans that are not candidates for conventional mutational screening. The RNAi Consortium, a public-private initiative of which Hahn is a member, has produced lentiviral vectors encoding RNAs that target around 20,000 mouse and human genes (four to five vectors per gene), and which are commercially available from consortium members Sigma-Aldrich (St. Louis, USA) and Open Biosystems (Huntsville, USA). Hahn is using high-content imaging to identify modulators of mitosis in a screen of arrays of cells transformed with lentiviral vectors that suppress the expression of individual protein kinases and phosphatases. Suppression of many of the kinases in this screen induces apoptosis, suggesting that they represent potential therapeutic targets. Unexpected hits include the kinases Yes, Tie1 and Met, which were not previously known to be involved in cell-cycle regulation.

Taking the proteomics route to identifying potential kinase substrates, Richard Polakiewicz (Cell Signaling Technology, Danvers, USA) described immunoaffinity/mass spectrometric profiling of tyrosine phosphorylation events in cancer cells, which has detected around 11,000 distinct phosphotyrosine sites across some 130 tumors and cancer cell lines. After treatment of seven non-small-cell lung cancer cell lines with the tyrosine-kinase inhibitor gefitinib (Iressa), to which some patients with lung cancer appear to respond favorably while many do not, there was a marked, but non-uniform, reduction in the levels of tyrosine phosphorylation. This technology has also been used to screen cell lines derived from patients with acute myelogenous leukemia (AML), and has revealed increased phosphorylation of Jak3 kinase substrates.

Many potential inhibitors inhibit multiple kinases, and elucidating the kinase-inhibition profiles of drug candidates will be necessary to predict their likely side effects. In this regard, Patrick Zarrinkar (Ambit Biosciences, San Diego, USA) described an assay system that characterizes a profile by the displacement of kinases from a solid support through competition for the ATP binding site. Not surprisingly,

imatinib (specific for Bcr-Abl, cKit, and the PDGF receptor) and staurosporine (a promiscuous kinase inhibitor), display radically different kinase-binding profiles, with most clinically relevant kinase inhibitors falling in between. Remarkably, even compounds optimized for powerful activity against one target - in this case the epidermal growth factor (EGF) receptor - can differ radically in their kinase-inhibitory profiles. Zarrinkar closed his talk with an account of how Ambit has used kinase-inhibitory profiling to optimize inhibitors of the receptor tyrosine kinase Flt3 with activity in a *nude* mouse xenograft model.

A problem of resistance

As with any drug, resistance against imatinib has emerged as a therapeutic problem. There are now more than 50 clinically characterized mutations that contribute to imatinib resistance in CML, some of which predate drug treatment, and new drugs are being sought to overcome this challenge. SGX Pharmaceuticals has developed the proprietary FAST (fragments of active structures) platform for the discovery of drug leads, which is based on X-ray crystallography of small fragments or scaffolds. I described its application to screening compounds against the 'wild-type' Bcr-Abl kinase and the imatinib-resistant Bcr-Abl Thr315Ile mutant in parallel to discover and optimize potent third-generation Bcr-Abl inhibitors. *In vitro* data show that compounds that inhibit Bcr-Abl and most of the clinically significant imatinib-resistant forms of this oncoprotein (including Thr315Ile) induce apoptosis in leukemic cells driven to proliferate by Bcr-Abl and inhibit phosphorylation of the Bcr-Abl substrate Crkl, but have no effect on normally dividing cells. SGX anticipates filing an investigational new drug application in 2007 to permit clinical development of a third-generation Bcr-Abl kinase inhibitor for treatment of imatinib-resistant CML.

Charles Sawyers (Howard Hughes Medical Institute and University of California, Los Angeles, USA) described lessons learned from his studies on resistance to imatinib and from recent clinical experiences with dasatinib, a second-generation Bcr-Abl inhibitor. Dasatinib, a dual Src/Bcr-Abl inhibitor, is active against many of these resistant kinases, with the important exception of the Thr315Ile mutant. Early clinical studies with dasatinib show the potential value of close monitoring of pharmacokinetic/pharmacodynamic correlates - which detail the behavior of the drug in the body: these demonstrated that leukemia patients who experienced only Src inhibition (without Bcr-Abl inhibition) did not respond with an amelioration of their cancer. Predictably, there were also no responses in patients with the Thr315Ile mutation. Moreover, disease progression was seen in patients who may have acquired the very same mutation. Sawyers also reported that in *in vitro* saturation mutagenesis studies of Bcr-Abl in the presence of dasatinib, the only imatinib-resistant mutation that emerged was Thr315Ile. In addition, the dasatinib selection yielded a small number of previously undescribed resistance mutations that are sensitive to imatinib. Taken together, these results make a case for up-front combination therapy for CML using agents with non-overlapping resistance profiles.

Visualizing drug action

David Piwnica-Worms (Washington University School of Medicine, St. Louis, USA) described strategies for pathway-specific molecular imaging *in vivo* that can be used in both basic research and clinical studies. He described the use of micro positron emission tomography (micro PET) combined with spatially determined expression of mutant forms of herpes simplex virus 1 thymidine kinase to detect trapped radiopharmaceuticals in groups of cells bearing the kinase. For whole-animal *in vivo* studies, bioluminescent imaging with firefly luciferase permits spatial mapping of a reporter gene within small animals such as mice. Applications of these techniques included imaging drug effects on the degradation of the NFκB inhibitor IκBα in the IKK/NFκB pathway in live mice, which enabled pharmacodynamic correlations of biochemical effect and drug levels; and imaging of two-hybrid studies of protein-protein interactions *in vivo*.

At the single-cell level, Gary Nolan (Stanford University School of Medicine, Stanford, USA) presented correlations between kinase activity and single-cell signaling metabolism in populations of mixed cell types analyzed using flow cytometry. Using monoclonal antibodies to both cell-surface proteins (to distinguish cell types) and intracellular phosphoproteins, phosphorylation and activation of the transcription factor Stat1 could be distinguished in subpopulations of B lymphocytes and T lymphocytes in a sample of murine splenocytes stimulated with interferon-γ. After showing this proof of concept, Nolan demonstrated how the approach provides insights into stratification of AML patients by examining the effects of cytokine stimulation of leukemic blast cells from individual patients on the levels of the phosphorylated forms of Stat3, Stat5, Stat6, and the kinases p38 and Erk1/2. Using these 'signaling maps', chemotherapy-insensitive patients with poor prognoses could be readily distinguished from those patients who did respond to standard chemotherapy. Such detailed characterizations may ultimately guide the co-administration of signal-transduction modulators with conventional chemotherapy to patients with poor prognosis signaling maps to improve treatment outcome.

Targeting receptor tyrosine kinases

Members of the EGF receptor family have proved important potential targets for anti-cancer drugs, and several inhibitors have been approved or are in clinical trials. They include the monclonal antibody Herceptin (trastuzumab), which targets the receptor Her, and small-molecule inhibitors. Gary Pestano (Ventana Medical Systems, Tucson, USA) discussed the evaluation of tissue-derived diagnostic phosphorylated

biomarkers in the EGF receptor pathway and presented the company's experiences with biomarker validation in the context of colorectal cancer progression. Upward and downward trends of various markers were documented as colorectal tumors underwent local and then distal metastasis. He presented a case study in which biomarker levels measured by the company's proprietary technology revealed a poor prognosis phenotype in a colorectal tumor of a Ventana employee, enabling the patient to elect to have adjuvant combination chemotherapy following radiation and colostomy.

Janet Dancey (National Cancer Institute, NIH, Bethesda, USA) described outcomes in clinical trials of EGF receptor inhibitors. Her receptors, which bind the EGF-like growth factor heregulin, were inhibited using either antibodies that target the extracellular portion of the receptor, or small molecules that target one or more kinase domains, or antisense oligonucleotides intended to suppress expression of a specific Her-family gene. Differences were observed in the toxicity and efficacy of antibodies versus small molecules that can be explained, at least in part, in terms of differences in mechanism of action, off-target activity, and pharmacokinetic behavior *in vivo*. Single-agent treatment of EGF receptor inhibitors (antibodies or small-molecule inhibitors) gave modest objective responses in lung, brain, head and neck, ovarian, esophageal, liver, and colon cancers. Studies of antibodies or small-molecule inhibitors in combination with cytotoxic chemotherapy or radiation have demonstrated survival benefits in some cases. Many additional randomized controlled trials are under way, and a clearer view of the utility of numerous single-agent and combination approaches to EGF-receptor-targeted therapy should emerge within the next two to three years.

Many cancer patients have mutations of EGF receptor genes, and Daniel Haber (Massachusetts General Hospital and Harvard Medical School, Boston, USA) presented analyses of the impact of EGF receptor mutations on individual sensitivity and resistance to tyrosine-kinase inhibitors. Opening with an anecdotal report of a Boston woman apparently cured of lung cancer with gefitinib, a small-molecule tyrosine-kinase inhibitor that has proved of very limited efficacy in most people, his presentation focused on studies of patients and model systems aimed at understanding why some patients respond to therapy with gefitinib and others do not. Most of the patients (40-80%) responding to small-molecule inhibitors of EGF receptor kinase domains exhibit kinase-activating mutations or gene amplification. In contrast, patients lacking mutations or amplification respond only rarely (10-15%). When compared with signaling by wild-type EGF receptors, signaling by mutant EGF receptors increases phosphorylation of the kinase Akt and the transcription factor Stat5 and downregulates Erk. Exposure of cells bearing mutant receptors to clinically achievable concentrations of small-molecule inhibitors of EGF receptors leads to apoptosis. Significantly higher drug concentrations are required to produce apoptosis in the context of the wild-type receptor. Regrettably, approximately half of the patients responding well to the small-molecule inhibitors do so for only a short time (3-6 months), after which relapse occurs, because the kinase domain has developed a drug-resistance mutation - Thr790Met, which is analogous to the imatinib-resistant Thr315Ile mutation in Bcr-Abl.

Mark Sliwkowski (Genentech, South San Francisco, USA) is examining the EGF receptor family with a view to designing new antibodies that target the receptor Her2, the target Herceptin. High-resolution X-ray crystallographic structures have provided detailed insights into Her2 heterodimerization and Her2-antibody interactions. These structures suggest alternative epitopes for targeting with novel antibodies. The Her2 sheddase (Mmp15, a membrane-linked metalloprotease) is responsible for cleavage of the extracellular component of the receptor, and Sliwkowski also described how resistance to Herceptin may be correlated with Mmp15 cleavage activity, which yields an activated, truncated form of the receptor lacking the epitope recognized by Herceptin. This hypothesis is currently being evaluated. Enzyme-kinetic analyses of Her2 with mutations in the kinase domain have explained the increased sensitivity of tumors bearing these mutant receptors to small-molecule inhibitors compared with tumors bearing a non-mutant receptor. These results suggest that high doses of small-molecule inhibitors will be crucial for treating patients with tumors driven to proliferate by non-mutant Her2.

Flt3 is a receptor tyrosine kinase that is important in leukocyte development and is being targeted as a possible treatment for AML. Donald Small (Johns Hopkins University School of Medicine, Baltimore, USA) described studies on Flt3 in AML patients. Individuals carrying the internal tandem duplication mutation in *FLT3* have considerably poorer responses to conventional induction chemotherapy with cytarabine and daunarubicin (the so-called 7+3 therapy) and correspondingly poor prognoses. Recent advances in preclinical characterization and clinical studies of Flt3 inhibitors were described in detail. The responses of patients with the internal tandem duplication mutation to therapy with a single Flt3 inhibitor are critically dependent on the blood levels of the drug. Current clinical studies on Flt3 inhibitors focus on relapsed AML patients who are receiving either high-dose cytarabine or a combination of mitoxantrone, etoposide and cytarabine. Ultimately, the best prospects for such inhibitors are likely to be in the setting of 7+3 induction chemotherapy for newly diagnosed AML patients with the Flt3 internal tandem duplication mutation.

Targeting of intracellular kinases

Many different intracellular protein kinases are components of mitogenic signaling pathways. David Solit (Memorial

Sloan-Kettering Cancer Center, New York, USA) described the targeting of the oncogenic kinase B-Raf, a mutant component of the Ras-Raf-Mek mitogen-activated protein kinase (MAPK) cascade, and also discussed how the presence of B-Raf mutations is correlated with the sensitivity of tumor cells to Mek inhibitors. Peter Lamb (Exelixis, San Francisco, USA) outlined high-throughput approaches to the discovery of phosphatidylinositol 3-kinase (PI 3-kinase) pathway inhibitors. An Exelixis compound (XL418) that inhibits Akt and P70S6 kinase blocks the growth of tumor xenografts in mice in a regime of either continuous or twice-weekly dosing. Combinations of XL418 with EGF-receptor inhibitors display synergy in tumor models, with dramatic increases in levels of apoptosis. Inhibitors of PI 3-kinase, Mek, and casein kinase 2 (CK2) were also described in a whirlwind tour of preclinical studies of various multi-targeted compounds. Lamb concluded that targeting multiple points within a given signal transduction pathway shows clear evidence of increased efficacy in model systems, which suggests various rational combinations of drugs for clinical intervention in the PI 3-kinase pathway.

Gary Gilliland (Harvard Medical School and Howard Hughes Medical Institute, Boston, USA) described the role of the protein kinase Jak2 in myeloproliferative disease (MPD), including diseases such as polycythemia vera, myelofibrosis, and essential thrombocytopenia, which have a cumulative prevalence of 80,000-100,000 in the US population. Gilliland also described the Harvard Myeloproliferative Study and presented the results of high-throughput sequencing of DNA from MPD patients, which frequently revealed an acquired somatic mutation, Val617Phe, in Jak2. This mutation occurs in the JH2 region of the polypeptide chain and renders Jak2 constitutively active, leading to uncontrolled proliferation of myeloid cells (leukocytes other than lymphocytes, megakaryocytes, and erythrocyte precursors) in response to stimulation with cytokines such as erythropoietin. Some individuals bearing the Jak2 Val617Phe mutation have excelled as athletes - in one celebrated case, achieving an Olympic gold medal in cross-country skiing - because of the advantage conferred by a 'naturally' elevated hematocrit. Competitors with normal Jak2 would have to resort to blood doping to achieve the same end. Gilliland discussed how the preclinical development of Jak2 inhibitors will be facilitated by the use of murine models of polycythemia vera.

Focusing on another pathway, Helen Piwnica-Worms (Washington University School of Medicine and Howard Hughes Medical Institute, St. Louis, USA) characterized the involvement of the Cdc25A/Chk1 cell-cycle regulatory pathway in normal and tumor cells. Two of the three mitotic protein phosphatases, Cdc25A and Cdc25B, are overproduced in a wide variety of human cancers. Phenotypic studies in Cdc25 knockout mice show that global inhibition of Cdc25 will almost certainly cause gastrointestinal toxicity in humans. Therefore, selective Cdc25 inhibition will probably be required for cancer therapy. Alternatively, ubiquitin-mediated degradation of Cdc25A could be induced by inhibiting its phosphorylation by the kinase Chk1. Piwnica-Worms described a phase 1 clinical trial in patients with solid tumors that is currently under way with the Chk1 kinase inhibitor 7-hydroxyl-staurosporine (UCN-01, a natural metabolite from a *Streptomyces* species), in combination with irinotecan, an approved topoisomerase 1 inhibitor used for the treatment of various cancers. Efforts are currently under way to understand the basis of the effects of UCN-01 effects in patients by analyzing the biology and biochemistry of signal transduction in the tumor cells.

Future prospects for kinase-targeted therapies

The final session of the conference was devoted to a free-ranging discussion of future directions in kinase-targeted cancer therapy. Paul Workman (Cancer Research UK, Sutton, UK) discussed drugging the cancer 'kinome' - all the kinases implicated in a given cancer - via optimization of combination therapies. Workman described the biological rationale underlying combination therapy for cancers, any one of which will have an estimated five to ten genetic abnormalities with perhaps two or more of these being critical for tumor growth and metastasis. Experience with inhibitors of PI 3-kinase and protein kinase B, both of which are *en route* to the clinic, were presented. Finally, the rationale for targeting the protein-folding chaperone Hsp90 as a means of achieving the equivalent of inhibiting multiple kinases simultaneously by interfering with their folding was discussed.

Kenneth Bair (Chiron Corporation, Emeryville, USA) concluded the meeting by presenting new paradigms for kinase inhibitor development. Offering the drug-hunter's point of view, Bair emphasized the importance of the early availability of X-ray structures (including co-crystal structures), multiple chemical frameworks, or scaffolds, for potential lead compounds, and cell-based assays and animal disease models. Lessons have been learned from CHIR-285 and CHIR-265, kinase inhibitors developed by Chiron. CHIR-285 inhibits at least ten distinct protein kinases, including growth factors and angiogenic factors, and has potent activity against more than half a dozen tumor types in xenograft models. CHIR-265 is a more focused inhibitor of mutant B-Raf and a modest number of other targets. Commenting on kinase inhibitor selectivity profiles, Bair was of the opinion that clinical trial design tends to be simpler for the more selective kinases. He also described how Chiron's database of kinase X-ray structures and kinase selectivity profiles has been combined with 150,000 compounds comprising the Chiron kinase-focused chemical library. Computational tools are being used to mine available data to identify lead scaffolds and compounds likely to exhibit the desired activity profile against a desired set of kinase targets. Subsequently, fewer than 10,000 compounds are evaluated for activity against the desired targets

and prioritized, permitting a small set of compounds from a single scaffold to be taken forward for hit-to-lead optimization and subsequent optimization of drug-like properties.

This exciting, wide-ranging meeting provided an up-to-the minute view of methodological and technical approaches to the challenges of discovering and developing new chemical entities that target protein kinases implicated in cell-cycle control, apoptosis, tumorigenesis and metastasis. Given that CML is one of only a very few malignancies caused by a single genetic lesion, namely the Philadelphia chromosomal translocation, the focus on targeted therapies should not be absolute. When this meeting reconvenes two years from now, we should see growing evidence of the benefits of combining targeted kinase inhibitors with one another, with other targeted therapies (such as Hsp90 inhibitors), and with cytotoxic antitumor therapies in the enormous number of multi-genetic-hit cancers represented by the acute leukemias, lymphomas, and solid tumors.